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Authors:
Thomas J. Fraites Jr, Mary R. Schleissing, **R. Andrew Shanely**, Glenn A. Walter, Denise A. Cloutier, Irene Zolotukhin, Daniel F. Pauly, Nina Raben, Paul H. Plotz, Scott K. Powers, Paul D. Kessler and Barry J. Byrne

Abstract

Pompe disease is a lysosomal storage disease caused by the absence of acid -1,4 glucosidase (GAA). The pathophysiology of Pompe disease includes generalized myopathy of both cardiac and skeletal muscle. We sought to use recombinant adeno-associated virus (rAAV) vectors to deliver functional GAA genes in vitro and in vivo. Myotubes and fibroblasts from Pompe patients were transduced in vitro with rAAV2-GAA. At 14 days postinfection, GAA activities were at least fourfold higher than in their respective untransduced controls, with a 10-fold increase observed in GAA-deficient myotubes. BALB/c and Gaa–/– mice were also treated with rAAV vectors. Persistent expression of vector-derived human GAA was observed in BALB/c mice up to 6 months after treat-ment. In Gaa–/– mice, intramuscular and intramyocardial delivery of rAAV2-Gaa (carrying the mouse Gaa cDNA) resulted in near-normal enzyme activities. Skeletal muscle contractility was partially restored in the soleus muscles of treated Gaa–/– mice, indicating the potential for vec-tor-mediated restoration of both enzymatic activity and muscle function. Furthermore, intra-muscular treatment with a recombinant AAV serotype 1 vector (rAAV1-Gaa) led to nearly eight times normal enzymatic activity in Gaa–/– mice, with concomitant glycogen clearance as assessed in vitro and by proton magnetic resonance spectroscopy.

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Correction of the Enzymatic and Functional Deficits in a Model of Pompe Disease Using Adeno-associated Virus Vectors

Thomas J. Fraites, Jr.,[1,2] Mary R. Schleissing,[1,2] R. Andrew Shanely,[3] Glenn A. Walter,[1,4] Denise A. Cloutier,[1,5] Irene Zolotukhin,[1,5] Daniel F. Pauly,[6] Nina Raben,[7] Paul H. Plotz,[7] Scott K. Powers,[3] Paul D. Kessler,[8,*] and Barry J. Byrne[1,2,5,†]

[1]*Powell Gene Therapy Center and Departments of* [2]*Molecular Genetics and Microbiology,* [4]*Physiology and Functional Genomics,* [5]*Pediatrics, and* [6]*Medicine, University of Florida College of Medicine, Gainesville, Florida 32610, USA*
[3]*Center for Exercise Science, University of Florida College of Health and Human Performance, Gainesville, Florida 32610, USA*
[7]*Arthritis and Rheumatism Branch, National Institute of Arthritis, Musculoskeletal, and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA*
[8]*Peter Belfer Cardiac Laboratory, Department of Medicine, Division of Cardiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, USA*

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Pompe disease is a lysosomal storage disease caused by the absence of acid *a*.-1,4 glucosidase (GAA). The pathophysiology of Pompe disease includes generalized myopathy of both cardiac and skeletal muscle. We sought to use recombinant adeno-associated virus (rAAV) vectors to deliver functional *GAA* genes *in vitro* and *in vivo*. Myotubes and fibroblasts from Pompe patients were transduced *in vitro* with rAAV2-*GAA*. At 14 days postinfection, GAA activities were at least fourfold higher than in their respective untransduced controls, with a 10-fold increase observed in GAA-deficient myotubes. BALB/c and *Gaa*$^{-/-}$ mice were also treated with rAAV vectors. Persistent expression of vector-derived human GAA was observed in BALB/c mice up to 6 months after treatment. In *Gaa*$^{-/-}$ mice, intramuscular and intramyocardial delivery of rAAV2-*Gaa* (carrying the mouse *Gaa* cDNA) resulted in near-normal enzyme activities. Skeletal muscle contractility was partially restored in the soleus muscles of treated *Gaa*$^{-/-}$ mice, indicating the potential for vector-mediated restoration of both enzymatic activity and muscle function. Furthermore, intramuscular treatment with a recombinant AAV serotype 1 vector (rAAV1-*Gaa*) led to nearly eight times normal enzymatic activity in *Gaa*$^{-/-}$ mice, with concomitant glycogen clearance as assessed *in vitro* and by proton magnetic resonance spectroscopy.

Key Words: glycogen storage disease type II, gene therapy, cardiovascular diseases, lysosomal storage diseases, musculoskeletal diseases

INTRODUCTION

Glycogen storage disease type II (GSDII; Pompe disease; MIM 232300) is an autosomal recessive disorder caused by a deficiency of the lysosomal enzyme acid *a*.-glucosidase (GAA; EC 3.2.1.20). GAA is responsible for the cleavage of *a*.-1,4 and *a*.-1,6 linkages in lysosomal glycogen, leading to the release of monosaccharides. A loss or absence of GAA activity leads to massive accumulation of lysosomal and cytoplasmic glycogen in striated muscle, causing contractile apparatus disruption, and ultimately resulting in contractile dysfunction and muscle weakness [1,2].

The most severe form of GSDII is characterized by rapidly progressing cardiac and skeletal myopathy, culminating in cardiorespiratory failure and death within the first 2 years of life [2–4]. There is a continuum of later onset disease that is accompanied by a spectrum of cardiac, skeletal, and smooth muscle myopathies, with a large number of patients succumbing to respiratory insufficiency as a result of diaphragmatic weakness [5]. Early attempts to treat GSDII, including a high-protein diet, *13*-adrenergic drugs, thyroid and steroid hormones, and bone marrow transplantation, have largely been unsuccessful [6,7]. Currently, no effective treatment is widely available, although clinical trials have begun to evaluate weekly infusion of exogenously produced, purified recombinant GAA [8–10].

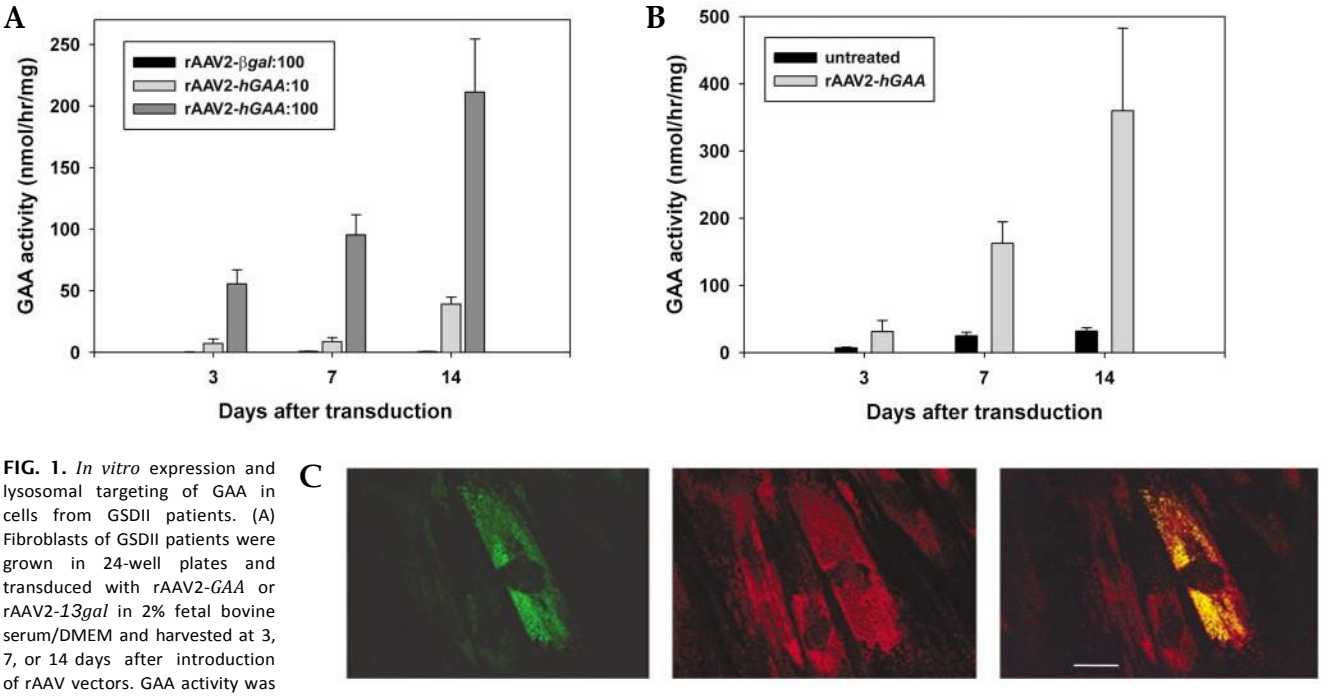

FIG. 1. *In vitro* expression and lysosomal targeting of GAA in cells from GSDII patients. (A) Fibroblasts of GSDII patients were grown in 24-well plates and transduced with rAAV2-*GAA* or rAAV2-*13gal* in 2% fetal bovine serum/DMEM and harvested at 3, 7, or 14 days after introduction of rAAV vectors. GAA activity was assayed as described. rAAV2-*13gal*:100, control cells transduced rAAV2-*13gal* at an MOI of 100; rAAV2-*GAA*:10 and rAAV2-*GAA*:100, cells transduced with a rAAV2-*GAA* at an MOI of 10 and 100, respectively. Bar graph represents mean ± SEM of GAA activities from independent triplicate cultures. (B) GSDII myoblasts were seeded in 24-well plates at a density of 2×10^4cells/cm^2 and cultured to confluence in growth medium, then harvested at 3, 7, or 14 days after infection with rAAV2-*GAA*. GAA activities were assayed as described. Untransduced, control cells with no rAAV vector; rAAV2-*GAA*, cells infected with rAAV2-*GAA* at an MOI of 10. Bar graph represents mean ± SEM of GAA activities from independent triplicate cultures. (C) Fibroblasts from a GSDII patient incubated with an anti-GAA antibody and a FITC-conjugated secondary antibody (left) 8 days after infection with rAAV2-*GAA*. The same cells were also incubated with an anti-LAMP-1 antibody and a rhodamine-conjugated secondary antibody (middle). A digitally merged FITC/rhodamine image (right) shows co-localization of the two signals in yellow, confirming that recombinant human GAA is sorted to the lysosomal compartment. Bar, 25 μm.

In normal cells, the 110-kDa precursor form of GAA is core-glycosylated in the endoplasmic reticulum and acquires mannose 6-phosphate (M6P) residues in the *cis* Golgi. Most of the GAA precursor molecules are sorted from non-lysosomal enzymes and transported to the lysosomal compartment via the M6P receptor. Successive cleavage of these precursors in the lysosome yields the 95-, 76-, and 70-kDa forms of GAA, all of which are catalytically active [11]. The remaining glycosylated GAA precursor molecules (about 10%) are not sorted to the lysosomes; instead, they enter the secretory pathway. This circulating fraction can be recaptured by M6P receptors on the surface of cell membranes, internalized, and directed to lysosomes. Therefore GAA can reach the lysosome from both intracellularly derived protein and through a secretion-recapture pathway [12–14].

These observations are critical to the development of genetic therapies for GSDII because they provide a rationale for the delivery of recombinant GAA in *trans* after transduction of a repository of producer cells. Gene replacement strategies offer the potential for long-term expression of a therapeutic protein after a single administration of vector, and early studies using viral vectors as a delivery system for *GAA* have yielded promising results. Zaretsky *et al*. [15] demonstrated effective transduction of GAA-deficient myoblasts with a recombinant retrovirus vector. We and others [16–18] have constructed recombinant E1-deleted adenoviral vectors carrying the human *GAA* cDNA (rAd-*GAA*) and have demonstrated their ability to direct expression of GAA both *in vitro*, in deficient fibroblasts, and *in vivo* after a single intramuscular or intracardiac injection [19]. Further investigation showed that deficient fibroblasts, after transduction with rAd-*GAA* *in vitro*, can serve as producer cells of recombinant GAA; that the recombinant protein can be secreted and recaptured by untransduced acceptor cells; and that the M6P receptor mediates this recapture process [19]. When a similar virus carrying the mouse *Gaa* cDNA (rAd-*Gaa*) was administered intravenously to a GAA-deficient mouse (*Gaa*$^{-/-}$) [20], secretion of the enzyme was demonstrated *in vivo*, with enzyme activity increased in both cardiac and skeletal muscle [19]. These results indicate that viral vectors have the potential to direct the synthesis of recombinant GAA in deficient cells.

The early onset and robust expression from recombinant adenovirus vectors establishes important proof of

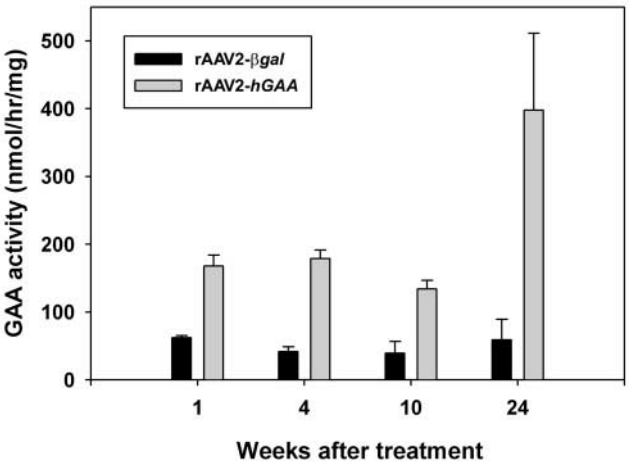

FIG. 2. Expression of recombinant human GAA in BALB/c mice after transduction with rAAV2-*GAA*. Adult mice were treated with 1 $\times$ 10^9 i.u. of rAAV2-*GAA* in the tibialis anterior muscle, muscle tissues were isolated at the time points indicated, and assayed for GAA activity. The bar graph represents mean ± SEM GAA activity in five animals (weeks 1 and 4) or four animals (weeks 10 and 24).

concept, however, adenovirus-mediated gene delivery elicits host immune responses in immunocompetent subjects, leading to transient expression of the transgene *in vivo* [21]. We have also constructed recombinant adeno-associated viral (rAAV) vectors that express human and mouse GAA (rAAV-*GAA* and rAAV-*Gaa*, respectively). Adeno-associated virus (AAV) has gained widespread attention in recent years as a potential vector for recombinant gene transfer in humans [22,23]. AAV is a nonpathogenic human parvovirus containing single-stranded DNA that requires helper viruses, such as adenovirus or herpesvirus, or other factors in order to replicate [24]. Recombinant AAV vectors contain none of the wild-type viral genes and retain only the characteristic inverted terminal repeats (ITRs), the only *cis*-acting sequences required for recombinant viral replication [25]. Neither recombinant nor wild-type AAVs cause any apparent disease in the host and lead to limited immunologic response. Early studies in our laboratory and others' [26–30] demonstrated that intramuscularly delivered rAAV is able to direct sustained expression of reporter and therapeutic genes. We report here the successful use of rAAV vectors to direct the synthesis of both human and murine GAA *in vitro* and *in vivo*, resulting in restoration of enzymatic activity and skeletal muscle contractility.

RESULTS

Human GAA Is Expressed and Is Enzymatically Active in GSDII Cells after *in Vitro* Transduction with rAAV2-*GAA*

We first examined the expression of recombinant human GAA in deficient fibroblasts and myotubes from patients with GSDII. Deficient fibroblasts have no GAA activity, whereas deficient myotubes retain 50 to 80% of the GAA activity of normal human myotubes. At 14 days after rAAV2-*GAA* transduction, GAA activity in deficient fibroblasts reached 30% of normal with a multiplicity of infection (MOI) of 10, whereas GAA activities of 150% normal were observed at an MOI of 100 (Fig. 1A). In deficient myotubes transduced with rAAV2-*GAA* at an MOI of 10, a 10-fold increase (360.0 ± 122.9 versus 32.0 ± 5.3 nmol/hour/mg) in enzymatic activity was observed 2 weeks after transduction (Fig. 1B). These data indicate that rAAV2-*GAA* is capable of restoring GAA activity in deficient cells *in vitro* in a dose-dependent manner.

To confirm that recombinant human GAA was being properly expressed and localized intracellularly, we probed for vector-derived human GAA protein in transduced, deficient cells. Immunofluorescent staining of human deficient fibroblasts transduced with rAAV2-*GAA* (Fig. 1C) showed that the protein is correctly targeted with a lysosomal distribution pattern. To confirm the lysosomal targeting of GAA, we tested for co-localization of GAA and LAMP-1, a specific marker for mature lysosomes. Positive staining for GAA (green; Fig. 1C, left) was coincident with the LAMP-1 staining (red; Fig. 1C, middle), indicating that GAA protein expressed from rAAV2-*GAA* is indeed transported to lysosomes (Fig. 1C, right).

In Vivo Delivery of rAAV2 Vectors Results in Stable, Long-Term Expression of Human or Mouse GAA in Mouse Muscle

To examine the efficiency and stability of rAAV2-mediated expression of GAA, we tested the vectors *in vivo* by injecting 1 $\times$ 10^9 i.u. of rAAV2-*GAA* into the tibialis anterior muscles of BALB/c mice. GAA expression was then assessed at 1 week, 4 weeks, 10 weeks, and 6 months after treatment (Fig. 2). We found that GAA enzymatic activity was increased over 150% in the tibialis anterior muscles at 1 week (168.1 ± 16.0 nmol/hour/mg treated versus 62.0 ± 3.1 control), and this level of activity was maintained or increased over 6 months, with the highest activities observed at the latest time point (397.9 ± 113.3 nmol/hour/mg). The control group, which was injected with rAAV2-*13gal*, showed no change in GAA enzymatic activity over the same period. These data demonstrate that the rAAV2 is capable of expressing GAA efficiently, and that the expression is stable for up to 6 months after a single intramuscular injection.

To provide further assurance that the observed enzymatic activities were not due to increased basal production in the BALB/c strain, we treated GAA mutant mice (*Gaa*$^{-/-}$) with rAAV2-*Gaa*. These mice have little or no residual GAA activity and have been shown to recapitulate many of the pathologic manifestations observed in human GSDII patients [20]. At 12 weeks after intramuscular delivery of 1 $\times$ 10^9 i.u. of rAAV2-*Gaa*, we observed normal levels of GAA

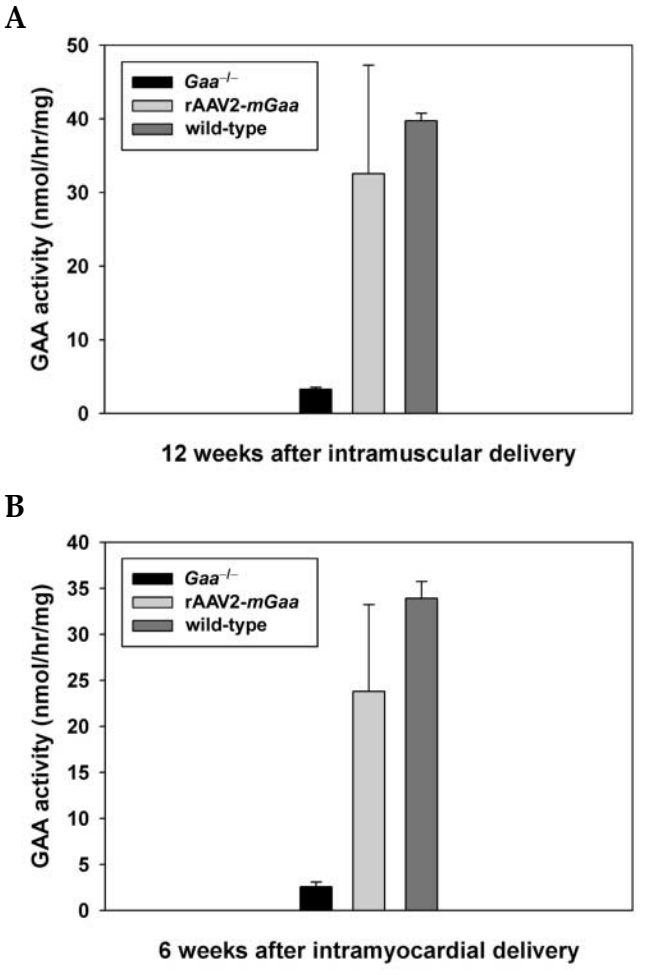

FIG. 3. rAAV2-*Gaa*-mediated transduction of skeletal and cardiac muscle in *Gaa*$^{-/-}$ mice. (A) Adult *Gaa*$^{-/-}$ mice were treated with 1 $\times$ 10^9 i.u. of rAAV2-*Gaa* in the quadriceps muscle. C57BL6/129SvJ controls and untreated *Gaa*$^{-/-}$ mice were sham-injected with sterile saline. Muscle tissues were isolated at 12 weeks after treatment and assayed for GAA activity. The bar graph represents mean ± SEM GAA activity for five mice in each group. (B) After intubation and a left thoracotomy, 1 $\times$ 10^9 i.u. of rAAV2-*Gaa* were directly injected into the left ventricular free wall of *Gaa*-mutant mice. Untreated *Gaa*$^{-/-}$ mice were sham-injected with sterile saline. Muscle tissues were isolated 6 weeks after treatment, assayed for GAA activity, and compared with untreated age-matched C57BL6/129SvJ (wild-type) mice. The bar graph represents mean ± SEM GAA activity for four rAAV2-*Gaa*-treated mice and five mice in each of the control groups.

enzyme activity in the mutant mice (32.6 ± 14.7 nmol/hour/mg), as compared with C57BL6/129SvJ control mice (39.7 ± 1.0 nmol/hour/mg; Fig. 3A). Similar results were obtained after intramyocardial injections (1 $\times$ 10^9 i.u. rAAV2-*Gaa*) in *Gaa*$^{-/-}$ mice (Fig. 3B), demonstrating that recombinant mouse GAA expression can be directed by rAAV2-*Gaa* in both skeletal and cardiac muscle.

Direct Intramuscular Delivery of rAAV2-*Gaa* Preserves Skeletal Muscle Contractile Force in Mutant Mice

We tested the contractile properties of soleus muscles of mutant and wild-type hybrid mice using isometric force-frequency relationships as an index of contractile function. *Gaa*$^{-/-}$ mice exhibited an age-dependent impairment of skeletal muscle function (Fig. 4, open squares), as evidenced by their decreased maximal tetanic force (16.71 ± 1.52 N/cm^2) at higher stimulation frequencies compared with the matched control strain (20.86 ± 1.88 N/cm^2; filled circles). This impairment is observed as early as 3 months of age (Fig. 4) and progressively worsens over the lifespan of the animal.

To test the effect of restoration of GAA activity on contractile dysfunction in *Gaa*$^{-/-}$ mice, we injected 2 $\times$ 10^9 i.u. of rAAV2-*Gaa* directly into the soleus muscles of 6-week-olds. Isometric force generation was tested 6 weeks later, at 3 months of age (Fig. 4, filled triangles). At the maximal stimulation frequency (200 Hz), treated *Gaa*$^{-/-}$ mice had intermediate contractile force (18.03 ± 2.05 N/cm^2) relative to untreated *Gaa*$^{-/-}$ and wild-type controls. Similar relationships in isometric tension were observed between wild-type, treated, and untreated *Gaa*$^{-/-}$ mice from 80 to 150 Hz, indicating some amelioration of the muscle function deficit over a range of physiologically relevant forces.

Treatment of *Gaa*$^{-/-}$ Mice with rAAV1-*Gaa* Leads to Rapid Overexpression of Mouse GAA and Glycogen Clearance

As rAAV2-mediated gene replacement led to wild-type levels of GAA enzymatic activity, we tested the ability of AAV serotype 1 (rAAV1) vectors to restore GAA activity as well. We injected 5 $\times$ 10^{10} total particles (as assessed by dot-blot analysis) of rAAV1-*Gaa* directly into the tibialis anterior (TA) muscles of 2-month-old *Gaa*$^{-/-}$ mice (n = 4), and the mice were sacrificed 2 weeks later. TA muscles were harvested, pooled, and homogenized. GAA activities (Fig. 5A) in treated *Gaa*$^{-/-}$ tissues (461.5 nmol/hour/mg protein) were nearly eight times wild type (65 nmol/hour/mg protein). Glycogen contents of TA muscles from untreated and treated *Gaa*$^{-/-}$ mice were 1.756 and 0.0219 μmol glucose/mg protein, respectively, compared with 0.128 μmol glucose/mg protein for wild-type mice. Proton nuclear magnetic resonance (^{1}H-NMR) spectra of perchloric acid extracts from the same treated and untreated tissues showed a pronounced glycogen peak for *Gaa*$^{-/-}$ mice and complete amelioration of glycogen accumulation in rAAV1-*Gaa* treated mice. These findings indicate a reversal of glycogen accumulation after transduction with rAAV1-*Gaa*.

Discussion

Pompe disease is due to a deficiency of GAA in all tissues, but is manifested primarily as cardiac and skeletal muscle weakness. Several mutant mouse models of GSDII have been developed [20,31], all of which mimic, according to one index or another, the myopathic phenotype associated

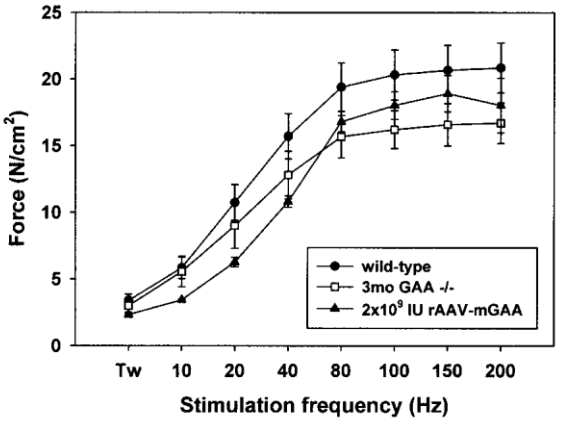

FIG. 4. Force-frequency relationships of intact soleus muscles after direct intramuscular delivery of rAAV2-*Gaa*. 2 × 10^9 i.u. of rAAV2-*Gaa* were directly delivered to the soleus muscles of 2-month-old *Gaa*$^{-/-}$ mice (n = 3). Muscles were isolated 6 weeks after treatment, tested for isometric force generation, and compared with untreated C57BL6/129SvJ (wild-type) (n = 6) and *Gaa*$^{-/-}$ mice (n = 5), respectively.

with GSDII. The *Gaa*$^{-/-}$ mouse used in these studies accumulates lysosomal glycogen and suffers from generalized skeletal myopathy, manifested in part by decreased locomotor activity [20]. Our objective was to achieve sustained restoration of GAA activity from direct intramuscular delivery to cardiac or skeletal muscle. Additionally, we sought to test the ability of vector-derived GAA to restore contractile function in muscle.

We constructed recombinant adeno-associated viruses that encode the full-length human *GAA* and mouse *Gaa* cDNAs and tested their ability to restore GAA activity *in vitro* in GAA-deficient fibroblasts and myotubes. Previous work had demonstrated the efficient transduction of these tissues with adenoviral vectors and indicated the utility of these cells as an *in vitro* system to evaluate potential therapies [15–18]. In this report, high-level expression of recombinant human GAA was achieved in both GAA-deficient fibroblasts and myotubes transduced *in vitro* with rAAV2-*GAA*, demonstrating that the human *GAA* cDNA is capable of directing synthesis of functional GAA protein in the context of rAAV.

In vivo experiments confirmed that rAAV2-*GAA* and rAAV2-*Gaa* were able to direct long-term enzymatic activity in treated mice. Stable expression of recombinant human GAA was observed for up to 6 months after a single intramuscular injection of rAAV2-*GAA*. In treated *Gaa*$^{-/-}$ animals, near-normal levels of mouse GAA activity were observed in skeletal muscle 12 weeks after administration of 10^9 i.u. (~ 10^{13} genomes/kg); concomitant improvement of muscle contractile function was observed in the higher frequency (that is, clinically relevant) stimulation range. These results are consistent with previous observations of efficient skeletal muscle transduction with rAAV vectors, as demonstrated with reporter [26–30] and therapeutic genes [26,28,32–34], as well as with genes encoding muscle contractile apparatus proteins [35,36] and that mutated in another lysosomal storage disease, mucopolysaccharidosis VII [37]. Likewise, intramuscular treatment with rAAV1-*Gaa* led to nearly eight times wild-type levels of GAA activity, demonstrating highly effective gene transfer in skeletal muscle with rAAV1, as has been observed previously in other models [38–40].

Our data indicate that rAAV2-*Gaa* delivery to the myocardium of *Gaa*$^{-/-}$ mice effectively restores GAA enzymatic activity, and is among the first examples of rAAV-mediated delivery of a therapeutic gene to the myocardium. In a previous report, Svensson *et al*. [41] described both direct intramyocardial delivery and intracoronary perfusion as potential methods for efficient rAAV delivery to the myocardium using *13*-galactosidase as a reporter. Recently, direct delivery of rAAV encoding recombinant vascular endothelial growth factor (rVEGF)

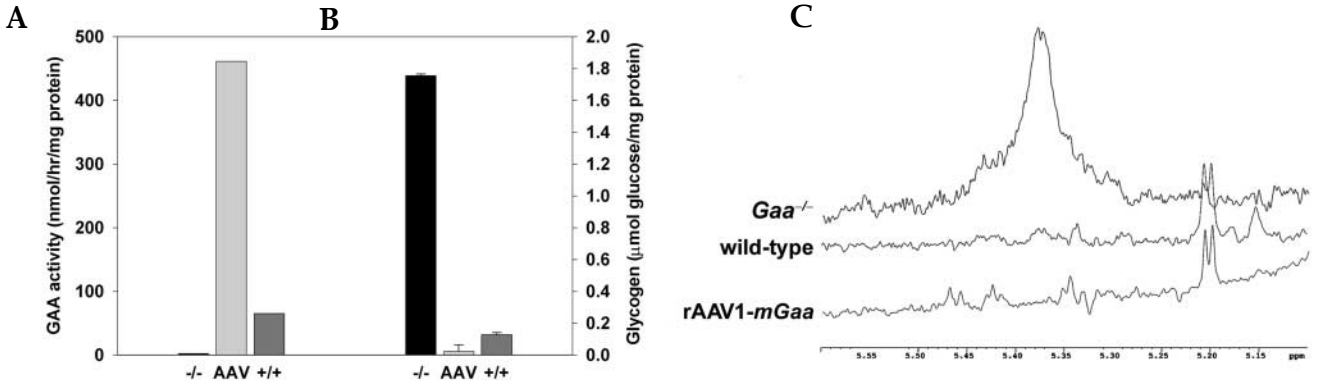

FIG. 5. rAAV1-*Gaa*-mediated transduction of skeletal muscle in *Gaa*$^{-/-}$ mice. (A) 5 × 10^{10} particles of rAAV1-*Gaa* were directly delivered to the tibialis anterior muscles of 2-month-old *Gaa*$^{-/-}$ mice (n = 4). Muscles were harvested, pooled, and homogenized 2 weeks after treatment and compared with untreated C57BL6/129SvJ (wild-type) and *Gaa*$^{-/-}$ mice, respectively. (B) *In vitro* glycogen content determination for the same muscle homogenates. (C) Stacked ^{1}H-NMR spectra from the same homogenates after perchloric acid extraction. Glycogen peaks are observed at 5.4 ppm.

has been used to induce neovascularization in an ischemic mouse model [42]. Likewise, Kawada *et al*. have demonstrated restoration of cardiac function in dystrophic mice [43] after direct intramural delivery of rAAV expressing *o*-sarcoglycan. The present work represents the first attempt to use adeno-associated virus vectors to address a metabolic cardiomyopathy, and investigations with regard to cardiac function after rAAV-mediated GAA restoration are ongoing.

The clinical heterogeneity of GSDII suggests that potential therapies must address the primary enzymatic deficiency in both skeletal and cardiac muscle. Numerous reports have indicated that circulating recombinant GAA, either purified protein or viral vector-derived, might serve as an effective method of delivery of GAA to muscle tissue. Several groups, including our own, have sought to take advantage of cell surface mannose 6-phosphate receptors to mediate the transport of circulating enzyme to the lysosomal compartments of individual cells. Studies with intravenous delivery of recombinant human GAA protein have indicated that this strategy may be beneficial in *Gaa*$^{-/-}$ mice and human subjects [8–10]. Our ongoing investigation will determine whether clinically relevant circulating enzyme concentrations can be sustained, particularly given the potential for neutralizing antibody responses to intravenously administered recombinant enzyme replacement therapy [44].

Data from *Gaa*$^{-/-}$ transgenic mice that express human GAA in a conditional, muscle-specific manner [45] indicate that several-fold overexpression of human GAA was required in order to achieve systemic restoration of GAA activity in non-muscle tissues. Given these data, the near-normal enzymatic activity observed after intramuscular delivery of rAAV2-*Gaa* (Fig. 3A) is not likely to provide sufficient circulating concentrations of GAA for systemic correction; in fact, no change in enzymatic activity was observed in the liver, heart, or uninjected (distal) skeletal muscles with the enzymatic activities reported here (data not shown). By contrast, GAA activities after intramuscular delivery of rAAV1-*Gaa* resulted in nearly eight times wild-type enzyme activity levels (Fig. 5), with concomitant clearance of glycogen from the injected tissue. Due to the relative size of the tibialis anterior muscle, we did not seek, in this report, to substantially increase circulating concentrations of recombinant GAA secreted from muscle. However, these data suggest that a larger, well-perfused muscle may be capable of serving as a production site for systemic therapy for GSDII. Further studies are underway to address this question.

We should note that the metrics used to analyze phenotypic correction of GSDII in both mice and humans are an area of active development in our laboratory and others. One potentially clinically important outcome is the co-localization of both active GAA enzyme and the clearance of lysosomal glycogen. So far, clinical trials with both intravenously delivered CHO- and transgenic rabbit milk-derived human GAA have led to variable increases in GAA enzymatic activity in skeletal and cardiac muscle, with achieved developmental milestones and reductions in cardiac mass. However, despite these promising observations, reductions in total tissue glycogen content have been reported in only one patient among the seven patients studied in two separate trials. We have recently begun to use magnetic resonance imaging and spectroscopy techniques in an effort to better understand the dynamics of glycogen clearance in vector-treated *Gaa*$^{-/-}$ mice (Fig. 5). The initial observations suggest that this technique will be very useful in longitudinal studies of treated mice. In addition to glycogen content, important morphological characteristics of skeletal muscle will also be evaluated in future studies.

We have described the use of recombinant adeno-associated virus vectors to correct the primary enzymatic defect in GSDII. These data suggest that adeno-associated virus-mediated gene transfer represents a feasible and potentially powerful strategy for the delivery of gene products to specific myopathic tissue. Given the short half-life of GAA *in vivo* (2–4 days [46]), gene therapy vectors with capabilities for both high-level and long-term persistent transgene expression will be required. We anticipate that direct delivery of rAAV will have broad applicability for the delivery of a variety of recombinant gene products to the failing heart and for the treatment of many inherited forms of skeletal and cardiac myopathy.

Materials and Methods

Molecular cloning of rAAV vectors carrying the human GAA and mouse Gaa genes. The human *GAA* and mouse *Gaa* cDNAs were constructed as described [19]. The full-length cDNAs were placed under the transcriptional control of the cytomegalovirus (CMV) immediate early promoter in the mammalian expression plasmid pCI (Clontech, Palo Alto, CA), yielding pCI-*GAA* and pCI-*Gaa*. The expression cassettes were then cloned into p43.2, a plasmid containing both of the AAV serotype 2 inverted terminal repeats (ITRs). The human vector plasmid, p43.2-*GAA*, was generated via *Eco*RI–*Xba*I, and p43.2-*Gaa* was similarly cloned via *Spe*I-*Mun*I. A control recombinant AAV vector plasmid (pAAV-*13gal*) carrying the gene encoding *Escherichia coli 13*-galactosidase under the transcriptional control of the CMV promoter has been described [26].

To confirm the enzymatic activity of recombinant GAA produced from p43.2-*GAA* and p43.2-*Gaa*, rAAV vector plasmids were transfected into COS-1 cells, and GAA activity was measured 72 h after transfection, as described below. An 8- to 10-fold increase in activity was observed after transfection with p43.2-*GAA* or p43.2-*Gaa*, compared with untransfected cells or cells transfected with pAAV-*13*gal (data not shown). The Johns Hopkins University DNA Analysis Facility confirmed DNA sequences for the two rAAV GAA plasmids using an automated sequencing protocol. Infectious rAAV2-*GAA*, rAAV2-*Gaa*, and rAAV2-*13gal* vectors were packaged and titered as described [47–49]. The current packaging protocol yields AAV particles that have a ratio of DNA-containing to infectious particles of < 100. SDS/PAGE and silver stain, infectious center assay, particle count, and electron microscopy were used to fully characterize high-titer vector stocks (up to 1×10^{11} infectious units (i.u.)/mL). Similar techniques were used to produce and isolate rAAV1-*Gaa* vectors.

Cell lines and in vitro and in vivo viral transduction. Cultured cells were maintained in 5% CO_2 at 37°C. GAA-deficient fibroblasts isolated from an infant with GSDII (GM04912) were obtained from the NIGMS Mutant Cell

Repository (Camden, NJ). Normal human skeletal muscle cells were obtained from Clonetics Corporation (Walkersville, MD).

LGM04912 cells were cultured in 24-well plates at a density of 1×10^5 in growth medium (GM; 20% [vol/vol] fetal calf serum (FCS) in DMEM). *In vitro* transduction with rAAV was performed in Opti-MEM and, after viral adsorption, cells were cultured in 2% FCS in DMEM. Normal and deficient human myoblasts were seeded in 24-well plates at a density of 2×10^4cells/cm^2 and cultured to confluence in GM. Once the cells reached confluence, differentiation medium (DM; 2% [vol/vol] horse serum in DMEM) was substituted to induce myoblast fusion and myotube formation. After 14 days of incubation in DM, myotubes were transduced with purified rAAV vectors in Opti-MEM. DM was reintroduced after viral adsorption. All media and sera were purchased from Life Technologies (Gaithersburg, MD).

All animal procedures were performed in accordance with the guidelines of the Animal Care and Use Committees of the Johns Hopkins University and the University of Florida College of Medicine. Delivery of recombinant viral vectors to mouse skeletal muscle has been described [26]. BALB/c mice were anesthetized with inhaled methoxyflurane, and 1×10^9 i.u. of rAAV2-*GAA* or rAAV2-*13gal* were injected into the tibialis anterior muscle after minimal exposure of the muscle via a single incision. For intramuscular rAAV2-*Gaa* experiments, rAAV2-*Gaa* (1×10^9 i.u.) was injected into the quadriceps muscle of *Gaa*$^{-/-}$ mice [20] using minimal exposure; mice were then sutured as described before. Control mice of the same genetic background (C57BL6/129SvJ) were injected with identical volumes of sterile saline.

To facilitate direct injection into cardiac muscle, adult *Gaa*$^{-/-}$ and control mice were anesthetized with an intraperitoneal (IP) injection of a ketamine/xylazine (100 mg/kg ketamine; 15 mg/kg xylazine) cocktail. Animals were placed in a supine position in a sterile surgical field. The trachea was exposed and a 22G catheter was introduced to facilitate ventilation using an SAR-830AP rodent ventilator (CWE, Ardmore, PA). The animal was ventilated at 110 breaths/min with a tidal volume of 0.2 cc/min. A left thoracotomy was performed, and the ribs were retracted to give full visualization of the left ventricle. Injections of 10 to 50 μL were carried out with a 29-gauge insulin syringe. The ribs and skin were closed, and the animal was weaned from the ventilator. All animals were monitored overnight for pain or distress and for 1 week or more for infection or other complications.

Assays of GAA activity and glycogen concentration. Enzymatic activity assays for GAA were performed as described [16]. Transduced tissue culture cells were harvested and lysed in a commercial lysis buffer (Analytic Luminescence Lab). Alternatively, harvested muscle tissues were homogenized in water, then subjected to three freeze-thaw cycles. Lysates were centrifuged and clarified supernatants were assayed for GAA activity by measuring the cleavage of the synthetic substrate 4-methylumbelliferyl-*a*.-D-glucoside (Sigma M9766, Sigma-Aldrich, St. Louis, MO) after incubation for 1 hour at 37°C. Successful cleavage yielded a fluorescent product that emits at 448 nm, as measured with a TKO100 fluorometer. Protein concentration was measured using a standard bicinchoninic acid method (Bio-Rad, Hercules, CA), with bovine serum albumin as a standard. Data are represented as nanomoles of substrate cleaved in 1 hour per milligram of total protein in the lysate (nmol/hour/mg). Glycogen concentration was assessed by measuring the amount of glucose released from tissue homogenates after treatment with amyloglucosidase as described [18,50].

Immunocytochemistry. For immunofluorescence microscopy, cells on coverslips were fixed with 50% methanol/50% acetone (vol/vol) at –20°C for 15 minutes. Samples were blocked with 50% FBS/50% PBS (vol/vol) for 1 hour at room temperature, then incubated for 1 hour at 25°C with a previously described rabbit-derived anti-human GAA antiserum [16], diluted 1:1000 in phosphate-buffered saline (PBS) with 50% FCS and 0.01% NaN_3. Cells were washed in PBS three times and incubated for 1 hour at 25°C with fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. The slips were again washed three times, mounted with an aqueous/dry-mounting medium (Biomeda, Foster City, CA), and examined with fluorescence microscopy. For localization of human GAA in the lysosomal compartment, transduced cells were fixed and probed simultaneously with a mouse monoclonal antibody recognizing human lysosome-associated membrane protein-1 (LAMP-1) and rabbit anti-human acid *a*.-glucosidase antiserum. Cells were incubated with tetramethyl rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG.

Perchloric acid extraction and ^{1}H-NMR spectroscopy. Mice were fasted overnight to lower background glycogen to minimal levels. Upon sacrifice, samples were prepared by rapid freezing in liquid nitrogen and pulverization into a fine powder. Liquid nitrogen was evaporated and the powder was transferred to a 15 mL polypropylene tube containing 3 mL 7% (vol/vol) perchloric acid in 50 mM NaH_2PO_4. The sample was vortexed repeatedly and centrifuged at 4°C and 4000 rpm for 15 minutes. The supernatant was transferred to a new tube and neutralized to pH 7.0 with 5 M potassium hydroxide, leading to precipitate formation. The precipitate was removed by centrifugation; the supernatant was transferred to a new tube, and paramagnetic metals and excess salts were removed by incubation with pre-washed Chelex beads at a 1:8 ratio for 20 minutes at 4°C. The mixture was filtered through a 0.22 μm filter and lyophilized overnight. Samples were resuspended in D_2O for spectroscopy.

Proton nuclear magnetic resonance (^{1}H-NMR) measurements were performed using a Bruker Avance 500 spectrometer with an 11.75 T Magnex. Spectra were collected under unsaturated conditions at 258C and pH 7.0 (TR = 5 seconds, sweep width = 6.666 KHz, pulse width = 5.5 μsec, number of averages = 256, number of points = 40K). Integrated areas and chemical shifts were referenced to the total creatine peak (3.0 ppm) for each sample.

Assessment of skeletal muscle function. Direct intramuscular injections of rAAV2-*Gaa* (2×10^9 i.u.) or lactated Ringer's were performed in the soleus muscle of *Gaa*$^{-/-}$ mice. After 6 weeks, the mechanical function of the muscles was assessed. *Gaa*$^{-/-}$ and C57BL6/129SvJ controls were anesthetized via IP injection of ketamine/xylazine. After reaching a surgical plane of anesthesia, the soleus muscles are surgically excised and placed in a cooled dissecting chamber containing Krebs-Henseleit solution, equilibrated with a 95% O_2/5% CO_2 gas mixture. The intact muscles are then vertically suspended between two lightweight Plexiglas clamps connected to force transducers (Model FT03, Grass Instruments, West Warwick, RI) in a water-jacketed tissue bath containing Krebs-Henseleit solution equilibrated with a 95% O_2 /5% CO_2 gas (bath ~ 37 ± 0.5°C, pH ~ 7.4 ± 0.05, osmolality ~ 290 mOsmol). Transducer outputs are amplified and differentiated by operational amplifiers and undergo A/D conversion for analysis using a computer based data acquisition system (Polyview, Grass Instruments).

In vitro contractile measurements begin with empirical determination of the muscle's optimal length (L_o) for isometric tetanic tension development. The muscle is field-stimulated using a stimulator (Model S48, Grass Instruments) along its entire length with platinum electrodes. Muscle length is progressively increased until maximal isometric twitch tension is obtained. Once the highest twitch force is achieved, all contractile properties are measured isometrically at L_o. The force-frequency relationship was examined as described [51,52].

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